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32. This begs the question as to why more riboregulators have not been found previously. Perhaps they had not been identified biochemically because of a predisposition towards searching for protein rather than RNA factors. They could be identified genetically, which was how *lin-4* and *let-7* were discovered (*1–3*); however, when compared to mutations in protein-coding genes, point substitutions in these short RNA genes would be less likely and perhaps less disruptive of function. Furthermore, mutations that map to presumed intergenic regions with no associated RNA transcript detectable on a standard RNA blot might be put aside in favor of other mutants.
33. WormBase is available on the Web at www.wormbase.org.
34. Sequencing traces (from the Sanger Center) representing 2.5- to 3-fold average coverage of the *C. briggsae* genome were obtained at www.ncbi.nlm.nih.gov/Traces.
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3 August 2001; accepted 14 September 2001

An Extensive Class of Small RNAs in *Caenorhabditis elegans*

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The *lin-4* and *let-7* antisense RNAs are temporal regulators that control the timing of developmental events in *Caenorhabditis elegans* by inhibiting translation of target mRNAs. *let-7* RNA is conserved among bilaterian animals, suggesting that this class of small RNAs [microRNAs (miRNAs)] is evolutionarily ancient. Using bioinformatics and cDNA cloning, we found 15 new miRNA genes in *C. elegans*. Several of these genes express small transcripts that vary in abundance during *C. elegans* larval development, and three of them have apparent homologs in mammals and/or insects. Small noncoding RNAs of the miRNA class appear to be numerous and diverse.

Small RNAs perform diverse functions within cells, including the regulation of gene expression (*1–4*). One class of regulatory RNA includes the small temporal RNA (stRNA) products of the genes *lin-4* and *let-7* in *Caenorhabditis elegans*. The *lin-4* and *let-7* RNAs are ~22 nucleotides (nt) in length, and are expressed stage-specifically, controlling key developmental transitions in worm larvae by acting as antisense translational repressors (*2–4*).

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lin-4 and *let-7* were identified by their mutant phenotypes (*2, 3*) and, until recently, were the only known RNAs of their class. However, the phylogenetic conservation of *let-7* RNA sequence and developmental expression (*5*), and the overlap between the stRNA and RNA interference (RNAi) pathways (*6, 7*), suggested that stRNAs are part of an ancient regulatory mechanism involving ~22-nt antisense RNA molecules (*8*).

To identify more small regulatory RNAs of the *lin-4/let-7* class in *C. elegans*, we used informatics and cDNA cloning to select *C. elegans* genomic sequences that exhibited four characteristics of *lin-4* and *let-7*: (i) expression of a mature RNA of ~22 nt in

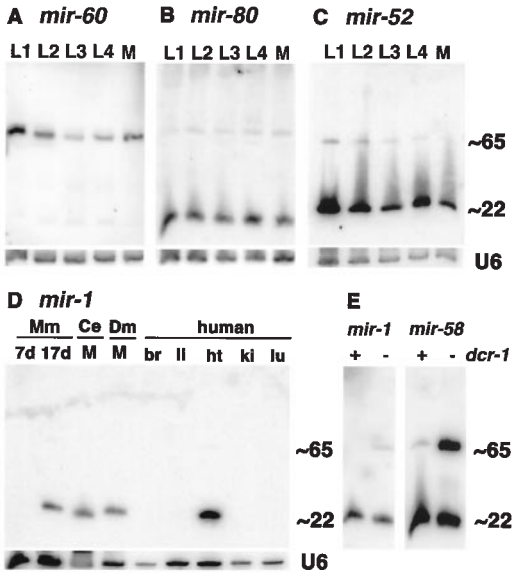

Fig. 1. Northern blots of small RNA transcripts. (A through C) Total RNA from *C. elegans* larvae (stages L1 through L4) or from mixed stage (M) populations were blotted and probed with oligonucleotides complementary to either the 5′ or 3′ half of the indicated transcript (*13*). U6 = the same filters were probed with probe to U6 snRNA as a loading control. (**A**) *mir-60* 5′ probe detects a transcript of ~65 nt. The ratio of L1 to L4 *mir-60* signal, normalized to U6, is about 5:1. The *mir-60* 3′ probe (not shown) detects a similar-sized species with a similar developmental profile. (**B**) *mir-80* 3′ probe detects a ~22-nt RNA expressed uniformly at all stages. (**C**) *mir-52* 5′ probe. The normalized *mir-52* signal is threefold greater in the L1 versus the L3. (**D**) *mir-1* 3′ probe detects a transcript of ~22 nt in total RNA from mouse (Mm) 17-day embryos, mixed-stage *C. elegans* (Ce), *Drosophila melanogaster* (Dm) mixture of embryo-larvae-pupae, and in a sample of human heart (ht) tissue. Other human tissue samples were brain (br), liver (li), kidney (ki), and lung (lu). (**E**) *mir-1* and *mir-58* probes to total RNA from mixed populations of wild-type (+) and *dcr-1(ok247)* (–) animals. An increase in the proportion of unprocessed ~65-nt precursor is observed in the *dcr-1* RNA.

length; (ii) location in intergenic (non–protein-coding) sequences; (iii) high DNA sequence similarity between orthologs in *C. elegans* and a related species, *Caenorhabditis briggsae*; and (iv) processing of the ~22-nt mature RNA from a stem-loop precursor transcript of ~65 nt (*2, 3*).

In an informatics approach to identifying candidate small regulatory RNAs, predicted *C. elegans* intergenic sequences that were also highly conserved in *C. briggsae* (*9, 10*) were analyzed using the RNA folding program "mfold" (*11–13*). Forty sequences were predicted by mfold to form a stem-loop similar in size and structure to *lin-4* and *let-7*. Probes complementary to these sequences were tested against Northern blots of total worm RNA (*13*), and three of them detected small RNA transcripts (Table 1 and Fig. 1A).

In a second approach, a cDNA library (about 1.6×10^6 independent *lambda* clones) was prepared from a size-selected (~22-nt) fraction of *C. elegans* total RNA (*14*) and sequence was obtained for 5025 independent inserts, representing 3627 distinct sequences (*13*). Some 386 of these sequences were represented by multiple (from 2 to 129) clones. Each of these multiple-hit cDNA sequences was compared using BLAST (*15*) to the NCBI database, and to approximately 800,000 raw sequence traces of *C. briggsae* genomic sequence (*16*). Single-copy cDNA sequences that corresponded to no previously known (or previously predicted) transcripts (*17*), and that were conserved in the *C. briggsae* genome, were analyzed using mfold for a predicted stem-loop structure. A total of 38 novel cDNA sequences fit these criteria, of which 13 were tested for expression by Northern hybridization; in all 13 cases, small transcripts (~22 nt and/or ~65 nt) were detected (Table 1 and Fig. 1). (The other 25 sequences have not been tested for expression.)

These 13 new genes identified by cDNA cloning, together with two additional genes from the informatics screen, were named *mir*, for microRNA (*18, 19*). All 15 of these miRNA genes appear to produce ~65-nt stem-loop transcripts (Fig. 2) that may be processed to ~22-nt forms by the same DCR-1/ALG-1/ALG-2 system involved with *lin-4* and *let-7* processing (*6, 7*). For the two RNAs that we tested (*mir-1* and *mir-58*), *dcr-1* activity was required for normal processing of the ~65-nt precursor (Fig. 1E). So in some cases, such as *lin-4* and *let-7*, the ~22-nt form is processed from the 5′ part of the stem (*6, 7*), and in other cases, such as *mir-1* and *mir-58*, from the 3′ part (Fig. 2), suggesting gene-specificity of miRNA processing and/or stabilization. For the three miRNA genes identified in our informatics screen (*mir-60*, *mir-88*, and *mir-89*), the longer stem-loop transcripts were detected by Northern blot, but ~22-nt forms were not detected, suggesting that their processing is inefficient, or is sharply restricted developmentally. For *mir-60*, 20-nt cDNA clones were identified, suggesting that *mir-60* is processed, but the mature form accumulates at levels below threshold for detection by Northern blot.

At least 10 of the 15 miRNAs vary in abundance during *C. elegans* larval development, perhaps reflecting roles for these particular genes in developmental timing (Table 1 and Fig. 1). *mir-1*, *mir-2*, and *mir-87* have apparent orthologs in mammals and/or insects (Table 1 and Fig. 1). *mir-1* is expressed tissue-specifically in humans (heart), and

Table 1. MicroRNA gene products in *Caenorhabditis elegans*.

Gene	Source*	Orthologs† Expression‡§‖	Development¶
mir-1	cDNA	C‡§‖ Dm‡ Hs‡	Unif
mir-2	cDNA	C‡§‖ Dm‡	L1
mir-42	cDNA	C‡‖	L1
mir-43	cDNA	C‡§‖	L1
mir-52	cDNA	C‡§‖	L1
mir-58	cDNA	C‡‖	Unif
mir-60	cDNA/In	C‖	L1
mir-62	cDNA	C‡§‖	L1
mir-72	cDNA	C‡‖	L1
mir-80	cDNA	C‡§‖	Unif
mir-81	cDNA	C‡‖	L1
mir-87	cDNA	C‡§‖ Dm Hs	L1
mir-88	In	C‖	Unif
mir-89	In	C‖	NT
mir-90	cDNA	C‡‖	L1

*Identified by screening a size-selected cDNA library (cDNA) or by informatics (In). †Predicted by BLAST (*15*) and mfold (*11, 12*). C, *C. elegans*; Dm, *Drosophila melanogaster*; Hs, *Homo sapiens*. ‡Expression of an ~22-nt RNA confirmed by Northern blot. §The ~22-nt transcript confirmed to be single-stranded using probes to both ends of the predicted stem loop. ‖Expression of an ~65-nt predicted stem-loop RNA confirmed by northern blot. ¶Stage of *C. elegans* larval development (L1 through L4) when the indicated transcript(s) appear most abundant. Unif, uniform expression; less than twofold change in level; NT, developmental profile not tested.

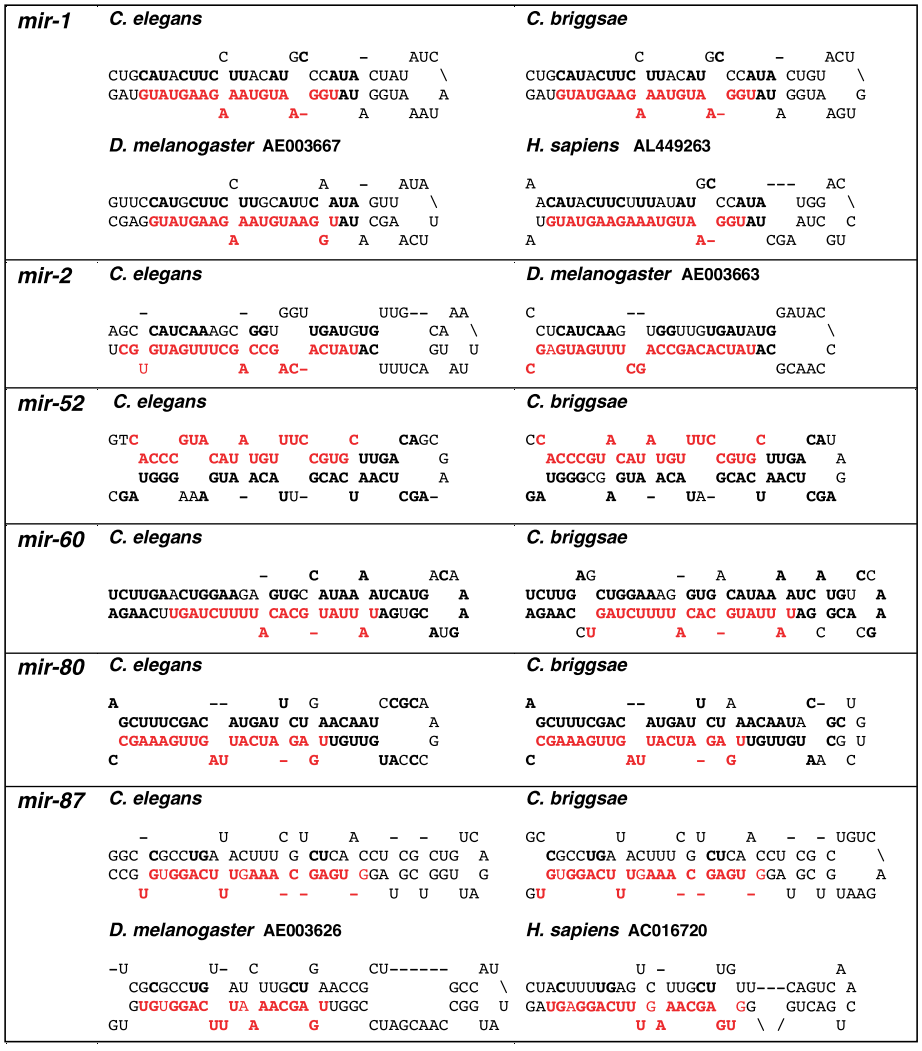

Fig. 2. Predicted secondary structures of stem-loop precursors of selected *C. elegans* miRNAs. Sequences of the ~22-nt mature small RNA are red, and were inferred from cDNA sequence, northern blots, and/or *C. elegans*::*C. briggsae* homology (Table 1). Phylogenetically conserved nucleotides are bold. The 5′ end is to the upper left.

stage-specifically in mouse embryogenesis (Fig. 1D). An evolutionarily conserved miRNA such as *mir-1* may have coevolved with its mRNA targets, and hence, could retain a similar developmental or physiological role in diverse taxa (*5*).

lin-4, *let-7*, and the 15 new miRNA genes described here are members of a gene family that could number in the hundreds in *C. elegans* (*18*) and other animals (*19*). To date, approximately 100 miRNA genes have been identified in worms, flies, and human cells (*18, 19*), and it is very likely that the screens conducted so far have not reached saturation. Therefore, additional *C. elegans* miRNAs can be identified using cDNA library sequencing. Also, continued application of whole-genome sequence alignment should identify additional new miRNAs, because this informatics approach complements the cDNA cloning. For example, using only a sample of the worm genome for *C. elegans*/*C. briggsae* alignment, we found two miRNAs (*mir-88* and *mir-89*) that were not represented in the size-selected cDNA library (perhaps due to absent or inefficient processing to the ~22-nt form).

This collection of new miRNAs exhibits a diversity in sequence, structure, abundance, and expression profile. If miRNA genes are as numerous and diverse as they appear to be, they likely occupy a wide variety of regulatory niches, and exert profound and complex effects on gene expression, development, and behavior. The challenge now is to determine the functions of the miRNAs, to identify potential antisense target mRNAs, and to characterize the consequences of their regulatory interactions.

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13 August 2001; accepted 18 September 2001

Single-Molecule Analysis of Chemotactic Signaling in *Dictyostelium* Cells

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Single-molecule imaging techniques were used to reveal the binding of individual cyclic adenosine 3′,5′-monophosphate molecules to heterotrimeric guanine nucleotide–binding protein coupled receptors on the surface of living *Dictyostelium discoideum* cells. The binding sites were uniformly distributed and diffused rapidly in the plane of the membrane. The probabilities of individual association and dissociation events were greater for receptors at the anterior end of the cell. Agonist-induced receptor phosphorylation had little effect on any of the monitored properties, whereas G protein coupling influenced the binding kinetics. These observations illustrate the dynamic properties of receptors involved in gradient sensing and suggest that these may be polarized in chemotactic cells.

Chemotaxis, the process by which cells move toward attractant molecules, operates in a range of biological processes including immunity, neuronal patterning, and morphogenesis. *Dictyostelium discoideum* cells display a strong chemotactic response to cyclic adenosine 3′,5′-monophosphate (cAMP), which is mediated by a cell surface receptor and G protein–linked signaling pathway (*1*, *2*). The signaling events downstream of the activated G proteins are initiated locally in the region of the chemotactic cell facing the higher concentration of attractant (cell anterior) even though receptors and G proteins are uniformly distributed on the cell surface (*3–7*). Because the regulatory mechanisms that localize these signaling events are essential for directional sensing (*3*), it is important to reveal the signaling activities of the cAMP receptors and their coupled G proteins in a chemotactic cell.

Recent progress in single-molecule detection techniques has allowed direct monitoring of signaling molecules on the surface of living cells (*8–10*). We extended this technique to real-time imaging of single fluorescent-labeled cAMP molecules bound to their receptors on living *Dictyostelium* amoebae. An orange fluorescent cyanine dye, Cy3, was conjugated to the 2′-OH of the ribose moiety (Cy3-cAMP; Fig. 1A) (*11*). Modification of this position had minimal effects on the binding affinity of the cAMP receptor (*12*), and Cy3-cAMP was functional as a chemoattractant for *Dictyostelium* cells by conventional chemotactic assays (*13*, *14*). The cells exhibited directional movements toward the tip of a micropipette containing 1 μM Cy3-cAMP solution (Fig. 1B). Treatment of cells with Cy3-cAMP or unlabeled cAMP also induced actin polymerization in the cells to a similar extent (*15*). Cy3-cAMP also bound to the cell surface uniformly, similar to the distribution of the cAMP receptors (Fig. 1C) (*4*). Binding of Cy3-cAMP to the cells was inhibited by addition of excess unlabeled cAMP, indicating specific binding of Cy3-cAMP to the cAMP receptors (Fig. 1D).

An objective-type total internal reflection fluorescence microscope (TIRFM) was used to achieve single-molecule imaging of Cy3-cAMP molecules on the basal surface of living cells (*8–10*). When Cy3-cAMP solution (1 nM) was added uniformly to cells, Cy3-

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Science **294** (5543), 862-864.
DOI: 10.1126/science.1065329